

How does familiarity in rhizobial interactions impact the performance of invasive and native legumes?

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Abstract

Mutualisms can be disrupted when non-native plants are introduced into novel environments, potentially impacting their establishment success. Introduced species can reassemble mutualisms by forming novel associations with resident biota or by maintaining familiar associations when they are co-introduced with their mutualists. Invasive Australian *Acacia* species in South Africa have formed nitrogen-fixing rhizobium mutualisms using both pathways.

Here we examined the contributions of novel vs familiar rhizobial associations to the performance of *Acacia saligna* across different soils within South Africa's Core Cape Subregion (CCR), and the concomitant impacts of exotic rhizobia on the endemic legume, *Psoralea pinnata*. We grew each legume with and without Australian *Bradyrhizobium* strains across various CCR soil types in a glasshouse. We identified root nodule rhizobium communities associating with seedlings grown in each treatment combination using next-generation sequencing (NGS) techniques.

Our results show that different CCR soils affected growth performances of seedlings for both species while the addition of Australian bradyrhizobia affected growth performances of *A. saligna*, but not *P. pinnata*. NGS data revealed that each legume associated mostly with their familiar rhizobial partners, regardless of soil conditions or inoculum treatment. *Acacia saligna* predominantly associated with Australian bradyrhizobia, even when grown in soils without inoculum, while *P. pinnata* largely associated with native South African *Mesorhizobium* strains.

Our study suggests that exotic Australian bradyrhizobia are already present and widespread in pristine CCR soils, and that mutualist limitation is not an impediment to further acacia invasion in the region. The ability of *P. pinnata* to sanction Australian *Bradyrhizobium* strains suggests that this species may be a good candidate for restoration efforts following the removal of acacias in CCR habitats.

Keywords

Australian acacias, *Bradyrhizobium*, Cape Floristic Region, familiar associations, legume-rhizobium mutualisms, novel associations, *Psoralea pinnata*, woody invader

Introduction

Novel abiotic and biotic conditions can act as strong barriers to the successful establishment of introduced non-native species (Blackburn et al. 2011). Many plants rely on mutualisms to complete their life cycles, but these are often disrupted when they are introduced into new environments (Richardson et al. 2000; Parker 2001). The re-establishment/replacement of effective mutualisms in the new range depends on the availability and diversity of resident mutualists as well as the level of interaction specificity of both the introduced plant and resident native mutualists (Parker 2001; Le Roux et al. 2017a). When non-native plants have generalist requirements, they could easily form novel and effective associations with (usually generalist) resident mutualists. For example, in the Galápagos Islands, generalist invasive plants have successfully infiltrated native seed dispersal networks by attracting generalist native bird and reptile seed feeders (Heleno et al. 2013). Conversely, specialist non-native plants may only persist if their historical (i.e. native-range; or highly similar) associations are maintained (Rodríguez-Echeverría et al. 2011). This can happen when they are co-introduced with their native-range mutualists (i.e., so-called familiar associations; Le Roux et al. 2017a) or when they encounter resident mutualists that are phylogenetically closely-related to their native-range mutualists. For example, introductions of many pine trees in the family Pinaceae initially failed in Southern Hemisphere countries due to a lack of compatible ectomycorrhizal fungi (EMF) (Policelli et al. 2019). Upon introduction of pine-specific EMF, however, the trees established successfully and in many instances became widespread invaders (Richardson et al. 1994). In fact, recent work suggests that pines that are more invasive are also more reliant on EMF mutualists than less invasive pines (Moyano et al. 2020, 2021), thus highlighting the need for mutualism reassembly for invasion success.

The legume family (Fabaceae) comprises approximately 19,500 species, many of which form mutualistic associations with nitrogen-fixing soil bacteria, called rhizobia. Rhizobia form nodules on their hosts within which they fix atmospheric nitrogen, converting it into forms that their legume hosts can utilise in return for carbon-rich photosynthates. Legumes are also over-represented in alien floras, with approximately 1,189 naturalised species globally (9% of the 13,168 world's naturalised alien plants; Van Kleunen et al. 2015; Pyšek et al. 2017). Range expansion by non-native legumes is constrained by the availability of effective rhizobial symbionts (Simonsen et al. 2017; Lopez et al. 2020), with generalist legumes being more likely to become widespread than those with specialist requirements (Klock et al. 2015; Harrison et al. 2018; Wandrag et al. 2020). Highly invasive legumes, therefore, often form associations

with different rhizobia in their native compared to non-native ranges (e.g., Australian *Acacia* spp., *Cytisus* spp., *Leucaena* spp. and *Robinia* spp. in Brazil – de Faria and de Lima 1998; *Acacia pycnantha* in South Africa – Ndlovu et al. 2013; *Trifolium* spp. in New Zealand – Shelby et al. 2016). Conversely, specialist legumes usually fail to colonise new areas when they are not co-introduced with their co-evolved rhizobia (Parker 2001; Gehlot et al. 2013). The genus *Mimosa* provides a useful example. In India, non-native *Mimosa pudica* could not effectively associate with rhizobial strains associated with native Indian *Mimosa* species and only successfully established invasive populations following the introduction of its familiar rhizobial mutualist from South America (Gehlot et al. 2013; Melkonian et al. 2014).

Legume-rhizobium co-introductions appear to be commonplace. For instance, Australian acacias and their rhizobia have been co-introduced into South Africa (Ndlovu et al. 2013; Le Roux et al. 2016; Warrington et al. 2019), New Zealand (Weir et al. 2004; Warrington et al. 2019), Portugal (Rodríguez-Echeverría 2010; Crisóstomo et al. 2013), and into their non-native ranges in Australia (Birnbaum et al. 2016). In South Africa, Keet et al. (2017) found a high level of host selection for Australian bradyrhizobia by acacias, with widespread and localised *Acacia* species predominantly associating with one or two co-introduced Australian *Bradyrhizobium* strains. Acacias in South Africa are also known to form novel associations with the resident Core Cape Subregion (CCR) rhizobia (e.g., Ndlovu et al. 2013). Together, this suggests that acacias are promiscuous host plants capable of forming both familiar (i.e., with co-introduced rhizobia) and novel (i.e., with resident native rhizobia) associations in their new ranges.

South Africa's CCR is renowned for its exceptional plant diversity, attributed, in part, to a complex mosaic of soil conditions (Linder 2003, 2005; Cowling et al. 2009; Manning and Goldblatt 2012). The region is home to an estimated 764 native legumes, of which 83% are endemic (Manning and Goldblatt 2012), and is also recognised as a hub for exceptionally high endemic rhizobial diversity, with all major rhizobial genera found in the region (Kock 2004; Elliott et al. 2007; Gerding et al. 2012; Hassen et al. 2012; Kanu and Dakora 2012; Beukes et al. 2013; Lemaire et al. 2015, du Preez 2019). Heterogenous soil conditions in the CCR are also perceived as important in determining legume community diversity and composition (Dludlu et al. 2018b), and, in turn, that of native rhizobial communities through host-plant selection and soil abiotic conditions (Lemaire et al. 2015; Keet et al. 2017; Dludlu et al. 2018a). *Bradyrhizobium* strains are not common associates of native CCR legumes (Lemaire et al. 2015). However, bradyrhizobia tend to have cosmopolitan distributions within soils (e.g., Le Roux et al. 2021) due to their low sensitivity to fluctuations in soil characteristics, such as pH, which, in turn, may benefit *Bradyrhizobium* specialists like introduced Australian acacias (Lange 1961; Lafay and Burdon 2001; Birnbaum et al. 2016; Le Roux et al. 2016; Keet et al. 2017; Kamutando et al. 2019). This low edaphic sensitivity, together with the presence of a compatible host, may therefore facilitate both the survival of exotic *Bradyrhizobium* strains and, subsequently, the successful colonisation by introduced acacias. Indeed, acacia invasions often result in localised enrichment of *Bradyrhizobium* strains in the CCR (Slabbert et al. 2014; Keet et al. 2017; Le Roux et al. 2018) which can lead to

homogenisation of rhizobial communities and lower native rhizobial diversity (Le Roux et al. 2018; Kamutando et al. 2019). This may facilitate the successful colonisation of other *Acacia* species as acacias often utilise the same bradyrhizobia interchangeably (Keet et al. 2017; Warrington et al. 2019) while negatively impacting native legumes, particularly when they are not compatible with *Bradyrhizobium* (e.g., Rodríguez-Echeverría et al. 2012). Decreased native rhizobial diversity and a concomitant increase in bradyrhizobia may also hamper the ability of some native legumes to sanction ineffective strains (Denison 2000; Westhoek et al. 2021).

Despite the wealth of information on acacias and their rhizobia in the CCR, it remains unclear how the presence of Australian rhizobia affects the growth performance of invasive acacias and co-occurring native CCR legumes. Here, we aimed to address this knowledge gap. A glasshouse experiment was set up to compare the performance of invasive *Acacia saligna* and native *Psoralea pinnata* grown in different uninvaded CCR soil types, with or without the presence of Australian *Bradyrhizobium* strains. Next generation sequencing (NGS) approaches were used to characterise the root nodule communities of both legumes under these different growth conditions. We hypothesised that the performance of *A. saligna* would be enhanced when forming familiar associations under treatments that received Australian bradyrhizobia inoculum while the performance of *P. pinnata* would be negatively impacted by the presence of exotic mutualists.

Methods

Study system

Acacia saligna (Labill.) Wendl., commonly known as Port Jackson willow, is native to south-western Australia and is invasive in many of the world's Mediterranean regions. Of the 15 invasive Australian acacias present in South Africa, *A. saligna* has the fifth largest distribution (Richardson et al. 2015). The species forms dense thickets with many devastating impacts on above- and belowground biodiversity and edaphic characteristics (Le Maitre et al. 2011). *Acacia saligna* is promiscuous and associates with a wide range of rhizobia, but, like most Australian acacias, is commonly nodulated by *Bradyrhizobium* strains (Marsudi et al. 1999; Lafay and Burdon 2001; Keet et al. 2017; Stępkowski et al. 2018).

Psoralea pinnata L., commonly known as fountain bush, is native to the south-western CCR and is found in a variety of fynbos vegetation types, particularly on acidic, nutrient-poor, sandstone-derived soils, or on richer shale soils (Bello et al. 2017). The species is predominantly nodulated by *Mesorhizobium* strains (Kanu and Dakora 2012; Lemaire et al. 2015), however, associations with *Paraburkholderia* (previously *Burkholderia*) and *Rhizobium* strains have also been documented (Kanu and Dakora 2012). Interestingly, *P. pinnata* has been introduced to western and eastern Australia where it has become naturalised, and has been identified as a potential invader, including in habitats where *A. saligna* naturally occurs (Stirton et al. 2015). No information

is currently available on the identity of rhizobia nodulating *P. pinnata* in Australia. In the CCR, it is frequently found growing in sympatry with Australian acacias (Staci Warrington, personal observation). Differences in the rhizobial associations of these two legumes, together with their sympatric distributions in Australia and South Africa, make them interesting systems to study the impact of familiar and novel mutualist associations on the performance of native and invasive species.

Soil collection

We collected soils from four pristine CCR areas to capture a range of abiotic conditions. As a fifth soil type, we also sampled soils directly beneath *P. pinnata* plants to capture the potential abiotic and biotic conditions induced by this species. These soils were collected during October 2018 across the Stellenbosch Winelands and Overberg districts of the CCR (see Suppl. material 1: Fig. S1 for site map and Suppl. material 1: Table S1 for site details).

The four non-*Psoralea*-conditioned soil types were collected at sites where neither *P. pinnata* nor *A. saligna* were present (other native legume species were observed at these sites). These sites were in the Grootbos Private Nature Reserve (sandy soils), Kogelberg Nature Reserve (sandy/loamy soils), Rustenberg Winery (clay soils), and Vergelegen Wine Farm (loamy soils). Within each site, soils were collected from four sampling points that were approximately 5m apart. The topsoil (the top 5cm of soil) was scooped aside and 25L of soil excavated at each sampling point. These were then mixed for each site and stored within a sterile storage container (i.e., 100L of soil in a single container per site). All soil sampling equipment was rinsed and sterilised with 70% ethanol between collections.

'*Psoralea*-conditioned soils' were collected directly beneath five different *P. pinnata* individuals spread across three different sites: Prawn Lake in Hermanus, Kogelberg Nature Reserve, and Vergelegen Wine Farm (Suppl. material 1: Fig S1, Table S1). Individual plants within each site were a minimum of 50 m apart, were over 1.5 m tall, and were part of a well-established population. The excavation procedure was the same as for the other four soil types. Twenty litres of soil were collected from within a 1m radius of each of the five shrubs, bulked and mixed thoroughly to make up 100L of soil per site in total.

All soils were separately sieved through a 4 mm mesh to remove any plant debris and rocks. All equipment were sterilised with 70% ethanol between sieving of individual soils. Soils were then returned to storage containers and stored at room temperature for a period of three months before commencing the glasshouse experiment.

Glasshouse experimental setup

We placed a layer of standard unsterilised store-bought drainage chips, followed by two litres of site-specific soil, into plastic gardening pots (18 cm diameter × 15.5 cm height), which were each placed onto a water-collecting saucer. This was done for a

total of 40 pots per soil type (five soil types; total $n = 200$). We chose to use whole soils (instead of soil inocula) to maintain all soil abiotic conditions that may favour native rhizobia (i.e., to which they are adapted), and to simulate the novel conditions under which co-introduced rhizobia would need to operate. All equipment used during this process was sterilised with 70% ethanol between potting of the different soil types. All pots were then watered with tap water until soils were saturated.

Seeds of *A. saligna*, collected from invasive CCR populations, were obtained from the Agricultural Research Council's Plant Protection Research Institute (ARC-PPRI) in Stellenbosch. *Psoralea pinnata* seeds, collected from populations across the Cape Peninsula in the CCR, were supplied by Silverhill Seeds in Kenilworth, Cape Town. Prior to planting, all seeds were surface-sterilised (Birnbaum et al. 2012), and scarified (*P. pinnata* – Siva et al. 2014; *A. saligna* – Rincón-Rosales et al. 2003). We planted four seeds of each species into 20 individual pots/soil type. Five weeks later we randomly removed all but one seedling if multiple seeds had germinated in each pot. In a few pots, no seeds germinated. To make up for these losses, extra seedlings removed from pots with high germination rates were transplanted into these pots, within the same species \times site \times inoculum treatment combinations (see Suppl. material 1: Table S2 for further details).

To ensure that rhizobial communities were still present in soils post-storage, we collected fresh soil from each site and added these to the pots as a soil inoculum (van de Voorde et al. 2012). Soil collections and sieving in the field were done as described above, except that only 60L of each soil type was collected. We added 0.2L of this fresh soil to the relevant pots (i.e., each pot containing a specific soil type received soil inoculum of the same soil type) for both species. This was done six weeks post-sowing once all seeds had germinated (Klock et al. 2015; Le Roux et al. 2018) and to ensure seedlings were tall enough to avoid being smothered by the added soil. We sterilised all equipment with 70% ethanol between additions of different soil inocula.

Australian *Bradyrhizobium* inoculum preparation

An Australian inoculum cocktail, consisting of five *Bradyrhizobium* strains that we previously isolated from *Acacia dealbata*, *A. decurrens*, and *A. melanoxylon* in Australia (Warrington et al. 2019; see Suppl. material 1: Table S3 for more details), was applied to the seedlings. These isolates are from the so-called Clade I *Bradyrhizobium* (*sensu* Stępkowski et al. 2018), an endemic lineage (Mishler et al. 2014) that houses the primary mutualists of many Australian *Acacia* species (Stępkowski et al. 2018; Le Roux et al. 2021). Therefore, although the strains we used were not isolated from *A. saligna*, they likely represent bacteria that are highly compatible with this legume. Indeed, many Australian acacias appear to share the same Clade I *Bradyrhizobium* strains interchangeably and with similar efficacy (Wandrag et al. 2013; Keet et al. 2017; Warrington et al. 2019). We grew these strains in separate Yeast Mannitol liquid broths in a shaking incubator (155 rpm) at 28 °C for a period of 5 days. We mixed 15mL of each strain, creating a rhizobial cocktail (75 mL) which was diluted in 1,425mL dH₂O to make up 1.5L of inoculum. Using a pipette, we added 5mL of this

inoculum to 10 of the 20 pots per species per soil type ($n = 10$ for each species \times soil type \times inoculum addition treatment combination). The remaining 10 pots for each soil type received 5mL sterile Yeast Mannitol broth that had been diluted in the same manner as the inoculum. Australian inoculum was first added seven weeks post-sowing as this allowed sufficient time (six weeks) for all seedlings to germinate (Klock et al. 2015; Le Roux et al. 2018) and one week for the bacterial communities added through the soil inoculum to establish. Inoculum addition was repeated four weeks later.

Glasshouse experiment protocols and measurements

All pots were randomly placed in a glasshouse exposed to ambient light and temperature conditions, and we randomised all pots weekly to minimise microclimate effects on seedling growth. Prior to soil inoculum addition, all pots were watered *ad libitum* two to three times a week with tap water. After adding the soil inoculum, a stringent watering system was put in place whereby we individually watered each pot every two days to minimise cross-contamination. All pots received the same amount of water. Randomisation took place prior to watering when saucers were dry to further minimise cross-contamination through spillage.

Plants were grown for a total of 17 weeks. Prior to harvesting plant material, we measured seedling height (defined as the length between the point where the stem exits the soil surface and the furthest apical meristem along the main stem). During seedling harvest we made every effort to minimise nodule loss and damage to seedling root systems. Each pot was gently tapped to loosen the soils from the sides of the pot. The seedling and the soil were then easily removed from the pot and placed onto a clean surface. Here, soils surrounding the root system were loosened further until they could gently be shaken from the roots. Any roots that had broken off during this process were collected. These roots, and those still attached to the plant, were rinsed in water to remove any remaining soil and tapped dry with tissue paper. Root nodules for each seedling were counted, removed, and placed into tubes containing silica gel for desiccation. Finally, we divided seedling biomass into root and shoot fractions and placed these into separate brown paper bags, followed by drying in an oven at 55 °C for one week. Dried shoot and root (excluding nodules) material, and desiccated root nodules were weighed separately. Altogether, the growth performance measurements included seedling height, seedling shoot dry biomass, seedling root dry biomass, seedling total dry biomass, and root:shoot ratios.

As a proxy for biological nitrogen fixation (BNF), we analysed $\delta^{15}\text{N}$ isotopic signatures (Lötter et al. 2014). Dried phyllode (*A. saligna*) and leaf (*P. pinnata*) material were removed after weighing of shoot material. This material was processed and sent to iThemba Labs (Pretoria, South Africa) for isotopic analysis (see Suppl. material 1: ESM1 for details). Generally, $\delta^{15}\text{N}$ values are expressed as parts per thousand deviation from the ^{15}N composition of atmospheric nitrogen (defined as 0‰; Mariotti 1983). The lighter isotope (^{14}N) is preferentially incorporated by nitrogenase during N-fixation. Consequently, $\delta^{15}\text{N}$ minima (i.e., extreme negative values) are reached

when plant-incorporated N is derived solely via BNF (i.e., atmospheric N), and values increase with increasing contribution of soil-derived N (Unkovich 2013). $\delta^{15}\text{N}$ values close to zero or negative are indicative of BNF while positive values suggest that NH_3 was predominantly assimilated from soil nutrient pools. These $\delta^{15}\text{N}$ measurements, together with the nodule count and nodule total dry biomass measurements, were used as proxies to estimate differences in nitrogen assimilation through BNF within specific soils in the presence and absence of Australian bradyrhizobia.

Statistical analyses of growth performance and BNF measurements

All statistical analyses were conducted in the R statistical environment (v3.4.4; R Core Team 2021) and separately for each legume species.

To investigate the effect of Australian inoculum and soil type on the overall growth performance (i.e., seedling height, seedling shoot dry biomass, seedling root dry biomass, seedling total dry biomass, root:shoot ratios), and BNF (i.e., number of nodules, nodule total dry biomass, $\delta^{15}\text{N}$) of the seedlings, we ran models using Australian inoculum (addition or no addition), soil type (Grootbos, Kogelberg, Rustenberg, Vergelegen, and *Psoralea*-conditioned), and their interaction as main effects. Factorial ANOVAs followed by Tukey HSD post-hoc tests were used for most of the performance and proxies of BNF measurements for both species, except for seedling total dry biomass for *A. saligna* seedlings, root:shoot ratio and nodule number for *P. pinnata* seedlings, and seedling root dry biomass for both species. Generalised linear models with a Gamma family data distribution (link = inverse) were used for seedling root dry biomass, seedling total dry biomass and root:shoot, and a generalised linear model with a negative binomial distribution for nodule number (See Suppl. material 1: Table S4 for details). The negative binomial data distribution family was chosen for nodule number to account for over-dispersion of data (Rodriguez 2013). We determined the overall effect size of Australian inoculum addition and soil type, and their significance, for all performance measures using the *Anova* function (type II sum of squares) in the *car* R package (Fox and Weisberg 2018). Finally, pairwise contrasts between levels of the main effects were determined using the *emmeans* function in the *emmeans* R package (Lenth et al. 2018).

To determine the relative contribution of the number of nodules to seedling growth performance and BNF under the two inoculum treatments; that is, the average gain in performance with increased nodulation (i.e., rhizobial efficacy), we regressed each growth and BNF measurement against nodule number (continuous predictor) and Australian inoculum addition treatment (categorical predictor) using generalised linear models (See Suppl. material 1: Table S4 for details on the data distribution families used for each measurement). Data from all soil types were combined for these analyses. We determined overall effect sizes and the significance of each main effect using the *Anova* function (type II sum of squares) for each performance and BNF measurement for each species, except for *A. saligna* seedling shoot dry biomass, seedling root dry biomass, seedling total dry biomass, root:shoot and nodule total dry biomass, for which type III sum of squares ANOVAs were used due to the significant interaction term.

DNA extraction and next-generation sequencing (NGS) of root nodule and inoculum rhizobia

To determine the identity and abundance of rhizobial strains within root nodules of *A. saligna* and *P. pinnata*, we pooled between 3–5 nodules from each seedling within a particular species \times soil \times inoculum treatment combination for each of the 20 combinations (i.e., 20 samples in total, each comprising 30–50 nodules). For DNA extraction, desiccated nodules were tissuelysed into a fine powder to create a homogenous mixture of nodule material. We extracted DNA from these mixtures using the DNeasy Plant Mini Extraction Kit (Qiagen, supplied by White Head Scientific, Cape Town, South Africa) according to manufacturer specifications.

To extract DNA of the Australian *Bradyrhizobium* isolates used in the inoculum, we grew all five strains from glycerol stocks in separate Yeast Mannitol broths in a shaking incubator (155 rpm) at 28°C until there was sufficient bacterial growth (indicated by a milky, turbid colour change). We extracted DNA from these cultures using the Sigma Gen-Elute Bacterial Genomic DNA kit (Sigma-Aldrich Co. LLC, USA), according to manufacturer specifications. Isolated DNA concentrations and quality were checked using a NanoDrop ND-1,000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). These samples were used as ‘reference’ strains in subsequent analyses.

We amplified the nodulation C (*nodC*) gene for NGS, using the primers nodCF12F (5'-CCG GAT AGG MTG GKB CCR TA-3') and nodCRI2R (5'-GTG CAC AAS GCR TAD RCC TTC AH-3'), with sample-specific barcodes in the forward primer. This gene has been successfully utilised for taxonomic identification of rhizobia in both the *alpha*- and *beta*-Proteobacteria (Le Roux et al. 2017b). Amplification and sequencing were performed at the Molecular Research LP next-generation sequencing service (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq instrument following manufacturer protocols. PCR conditions and sequencing protocols can be found in Suppl. material 1: ESM2.

NGS bioinformatics

NodC sequences were joined, and sequences < 150bp in length or with ambiguous base calls were removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences were denoised; unique sequences identified with sequencing or PCR point errors were removed; and chimeras removed, yielding zero-radius Operational Taxonomic Units (zOTUs).

Since no reference database exists for *nodC* sequences, each zOTU was blasted against the NCBI's GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast>) to determine its potential taxonomic identity. All non-nitrogen-fixing bacteria were removed from the dataset so that only rhizobia were considered in subsequent analyses. We clustered the remaining zOTUs at 97% DNA sequence similarity via the nearest-neighbour algorithm, based on pairwise sequence similarity distances calculated with the Needleman-Wunsch algorithm in mothur v1.44.1 (Schloss et al. 2009).

We found many low-abundance OTUs (<100 sequence reads/sample). Therefore, the relative abundance of each OTU within individual samples (i.e., each species × soil × inoculum treatment combination) was calculated and all rare OTUs, that is, those making up less than 5% of the cumulative abundance per sample for all samples, were removed from the dataset. This resulted in a final dataset comprising ten OTUs that occurred at a relative abundance of > 5% in at least one sequenced sample.

Phylogenetic analysis

Blast results indicated that most of our ten OTUs belonged to the genus *Bradyrhizobium*. In order to determine the possible geographic origin of these strains, we obtained additional *nodC* sequence data previously generated using the same approaches outlined above (i.e., the same primers and NGS platform) of *Bradyrhizobium* strains isolated from the root nodules of invasive acacias (Keet et al. 2017) and acacia-invaded soils in South Africa (Le Roux et al. 2018), as well as from root nodules of the *Acacia* species (from which some of our inoculum strains were isolated) in Australia (*A. decurrens* and *A. melanoxylon*; Urbina and Klock, unpublished). We downloaded *nodC* sequence data from GenBank for *Bradyrhizobium* strains previously isolated from native CCR legumes (Lemaire and Muasya, unpublished). We also included one *Mesorhizobium* *nodC* sequence as an outgroup. These additional sequence data were trimmed and aligned with our data using Clustal W in BioEdit (Hall 1999).

The best-fit nucleotide substitution model for the aligned dataset was determined using JModelTest (Posada 2008) and Akaike information criterion (Akaike 1973). The HKY + G + I (Hasegawa et al. 1985) model was identified as the best fit model. We then used MEGA X (Kumar et al. 2018; Stecher et al. 2020) to reconstruct a phylogeny using this model and maximum likelihood search criteria. Bootstrap values were calculated using the majority rule consensus method to assess topological support of the phylogeny.

OTU comparisons between treatments

To investigate the prevalence of the Australian inoculum *Bradyrhizobium* strains in association with *A. saligna* seedlings, the relative abundances of dominant inoculum OTUs were compared between inoculum treatments. These comparisons were only done for SW OTU1 and SW OTU6 as these were the only OTUs present within the reference samples with a relative abundance > 5% (see Results). We combined the relative abundance data for all soils and compared these between the two inoculum treatments using a paired t-test and a Wilcoxon signed-rank test for SW OTU1 and SW OTU6, respectively.

The relative abundances of each of the ten individual OTUs were compared between the different species × Australian inoculum addition treatment combinations for all soil types combined. This was done to determine whether *A. saligna* and *P. pinnata* differed in their rhizobial associations and whether these associations differed in the presence of the exotic *Bradyrhizobium* (i.e., inoculum addition). We performed these comparisons

using a permutational multivariate analysis of variance (PERMANOVA) in the *vegan* R package (Oksanen et al. 2013). A distance matrix for relative abundance data of all ten OTUs was developed following the Bray-Curtis dissimilarity method using the *vegdist* function and used this matrix as the response variable in the PERMANOVA with Australian inoculum addition treatment (inoculum added or not added) and host species (*A. saligna* and *P. pinnata*), as well as their interaction, as main effects. The PERMANOVA was run using the *adonis2* function with 999 permutations. We performed post-hoc analyses using the *simper* function to elucidate which OTUs were contributing most to any dissimilarities in the nodule rhizobial community composition. All functions form part of the *vegan* R package.

Results

Australian inoculum addition and soil type

As growth performance measurements were frequently significantly correlated for both species (results not shown), we only report on seedling total dry biomass (significantly correlated with seedling root and shoot biomasses and seedling height) and root:shoot ratios. Similarly, only nodule number (which correlated with nodule total dry biomass) and $\delta^{15}\text{N}$ are reported as proxies of BNF (also see Suppl. material 1: Tables S5, S6; Figs S3, S4). Increases in seedling total dry biomass accompanied by low root:shoot ratios are interpreted as advantageous as this indicates that plants invested more heavily into shoot biomass than root biomass. This is often due to a higher nutrient availability either through increased soil nutrient availability or through effective rhizobial associations (Friel and Friesen 2019).

There was a significant inoculation effect leading to increased nodule formation in *A. saligna* seedlings ($F_{(1)} = 5.638$, $p = 0.0201$; Suppl. material 1: Table S5). However, this did not translate into differences between inoculation treatments within each soil type (Fig. 1) and there was a significant interaction between inoculation and soil type for *A. saligna* $\delta^{15}\text{N}$ values ($F_{(1)} = 2.507$, $p = 0.0488$; Fig. 1; Suppl. material 1: Table S5). Counterintuitively, this was primarily driven by an increase in $\delta^{15}\text{N}$ values in *Psoralea*-conditioned soils for those seedlings that received Australian inoculum (Fig. 1). In contrast to *A. saligna*, there was never a significant Australian inoculum addition effect nor a significant interaction between inoculum addition and soil type for any *P. pinnata* growth performance and $\delta^{15}\text{N}$ measurements.

Soil type significantly influenced all growth performance and $\delta^{15}\text{N}$ measurements of both species (Suppl. material 1: Table S5). Both species appeared to have significantly higher total biomass and nodule numbers, and lower $\delta^{15}\text{N}$ values when grown in soils from Rustenberg and *Psoralea*-conditioned soils (Fig. 1). Root:shoot ratio responses were largely similar across all five soil types for both species, with differences in biomass allocation only manifesting between different inoculum treatments of the same (*A. saligna* in *Psoralea*-conditioned soils) or different (*P. pinnata* in Grootbos, Rustenberg, and Vergelegen) soils (Fig. 1).

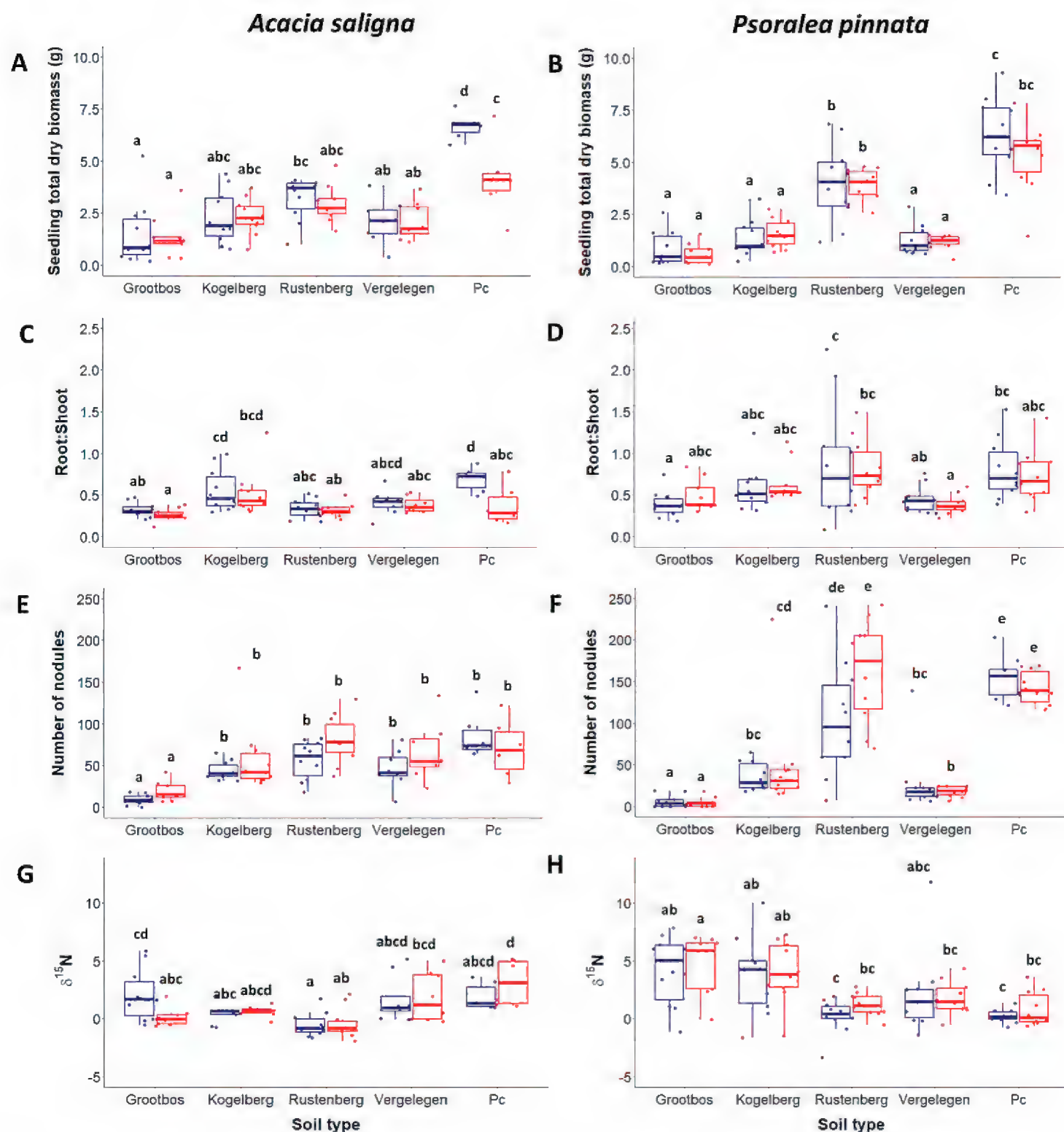


Figure 1. Comparisons of seedling performances between Australian inoculum addition and soil type treatment combinations. Seedling total dry biomass (**A, B**), root:shoot ratio (**C, D**), number of nodules (**E, F**) and $\delta^{15}\text{N}$ (**G, H**) measurements for *Acacia saligna* (left) and *Psoralea pinnata* (right) for each site (Grootbos, Kogelberg, Rustenberg, Vergelegen and *Psoralea*-conditioned (Pc) soils) by inoculum treatment (red – Australian inoculum added; blue – no inoculum added) combination.

Rhizobia efficacy

For *A. saligna*, nodule number was a significant predictor of seedling total dry biomass ($\chi^2_{(1)} = 43.862$; $p < 0.0001$) and root:shoot ratios ($\chi^2_{(1)} = 14.8465$; $p = 0.0001$), both of which increased with increasing nodulation, and $\delta^{15}\text{N}$ values ($\chi^2_{(1)} = 4.2034$; $p = 0.0403$), which decreased with increasing nodulation (Fig. 2; Suppl. material 1: Table S6). While Australian inoculum addition on its own was not significant for

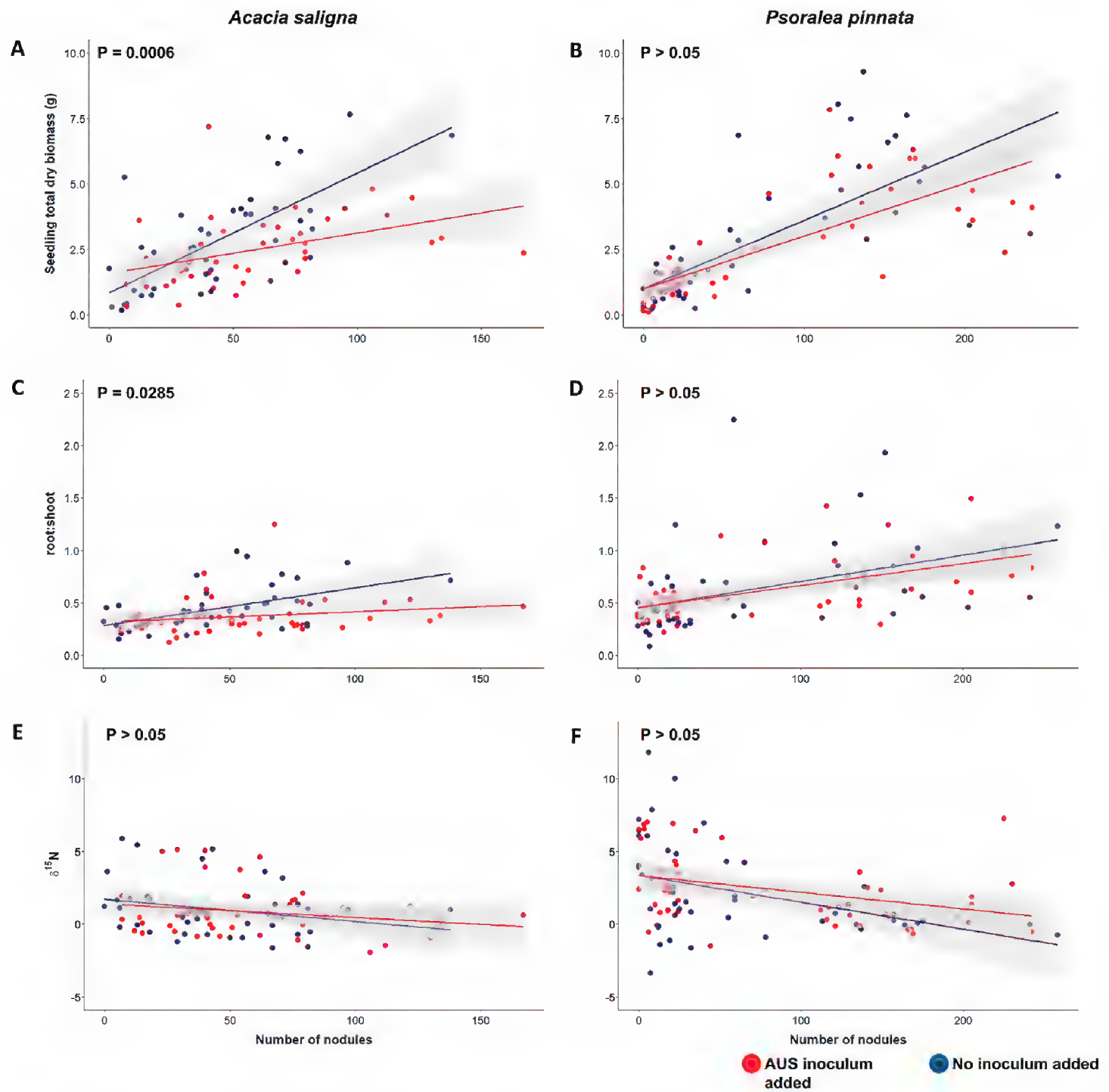


Figure 2. Comparisons of rhizobial efficacy between inoculum addition treatments. The contribution of nodules to seedling total dry biomass (**A, B**), root:shoot ratios (**C, D**) and $\delta^{15}\text{N}$ (**E, F**) of *Acacia saligna* (left) and *Psoralea pinnata* (right) for all sites combined and the influence of inoculum treatment (red – Australian inoculum added; blue – no inoculum added) on each. P-values for interaction terms (i.e., nodule number*inoculum) are provided.

any of the measurements, there were significant interactions between the number of nodules and Australian inoculum addition for *A. saligna* seedling total dry biomass ($\chi^2_{(1)} = 11.692$; $p = 0.0006$) and root:shoot ($\chi^2_{(1)} = 4.7948$; $p = 0.0285$), but not for $\delta^{15}\text{N}$ ($\chi^2_{(1)} = 0.2406$; $p = 0.6237$). *Acacia saligna* seedlings that did not receive inoculum gained more total biomass and root:shoot ratios than those seedlings that did receive inoculum. That is, for a given number of nodules formed, these values were higher for uninoculated seedlings than for inoculated seedlings, as indicated by the difference in slope for these two treatments (Fig. 2; Suppl. material 1: Table S6, Fig. S4). This increase in total biomass for uninoculated *A. saligna* seedlings is likely driven by

an overall higher investment in belowground rather than aboveground growth for all soil types, as shown by the root:shoot ratios of uninoculated seedlings tending to be higher than inoculated seedlings (Fig. 1), though these were non-significant.

For *P. pinnata* seedlings, inoculation as a main effect, as well as the interaction between the number of nodules formed and Australian inoculum addition, were non-significant for both measures of seedling growth performance and the $\delta^{15}\text{N}$ values (Suppl. material 1: Table S6). Only nodule number as a main effect was significant for all measurements ($p < 0.0001$ in all instances; Suppl. material 1: Table S6).

NGS Bioinformatics and phylogeny

After data quality-checking, the *nodC* dataset generated 272 zOTUs. Removing zOTUs representing non-nitrogen-fixing bacteria (34.6% of zOTUs), followed by clustering the remaining zOTUs at 97% DNA similarity level, and the removal of singleton/doubleton OTUs (leaving a total of 45 clustered OTUs) and OTUs with $< 5\%$ relative abundance per sample (77.8% of clustered OTUs) for all samples, resulted in 943,739 sequences representing ten OTUs.

Blast results for these OTUs indicated that they belonged to the genera *Bradyrhizobium* (five OTUs), *Mesorhizobium* (four OTUs), and *Rhizobium* (one OTU) (Suppl. material 1: Table S7). Of these, only two OTUs (SW OTU1 and SW OTU6) were present in the reference samples used in the Australian inoculum with a relative abundance $> 5\%$. These blasted to *Bradyrhizobium* sp. CPI240 and *Bradyrhizobium* sp. CPI241, respectively, previously isolated from *Acacia* species in Australia (Barrett et al. 2016). SW OTU1 and SW OTU2 were the dominant strains isolated from nodules of *A. saligna* and *P. pinnata*, respectively, with blast results identifying SW OTU2 as being closely related to *Mesorhizobium* sp. 969n9 previously isolated from South African legumes (Lemaire & Muasya, unpublished) (Suppl. material 1: Table S7). Blast results also revealed that *A. saligna* and *P. pinnata* associated with native CCR *Mesorhizobium* strains (SW OTU17) in Grootbos soils, and Australian *Bradyrhizobium* strains (SW OTU1) in Vergelegen soils, respectively. These are the only instances of novel associations identified in this study (Fig. 3).

The *Bradyrhizobium nodC* phylogeny yielded many unsupported nodes, likely because of the short length (312 bp) of the NGS reads (Fig. 4). However, it provided high support for two distinct clades, one including *Bradyrhizobium* strains previously isolated from native CCR legumes and the other including *Bradyrhizobium* from this study and strains previously isolated from acacia-invaded soils (JLR OTUs in Fig. 4; Le Roux et al. 2018) and acacia-associated root nodules in South Africa (JHK OTUs in Fig. 4; Keet et al. 2017), and acacia root nodules in Australia (HU_MG accessions in Fig. 4; Urbina and Klock, unpublished). Several of our OTUs (i.e., SW OTUs) clustered with these previously reported acacia OTUs with high support. Specifically, the dominant *Bradyrhizobium* OTU found in this study, SW OTU1, clustered with the dominant OTUs identified by Keet et al. (2017), Le Roux et al. (2018) and Urbina and Klock (unpublished). The second most abundant *Bradyrhizobium* OTU in our study, SW OTU6, clustered with an abundant OTU identified by Keet et al. (2017) and Urbina and Klock (unpublished) (Fig. 4).

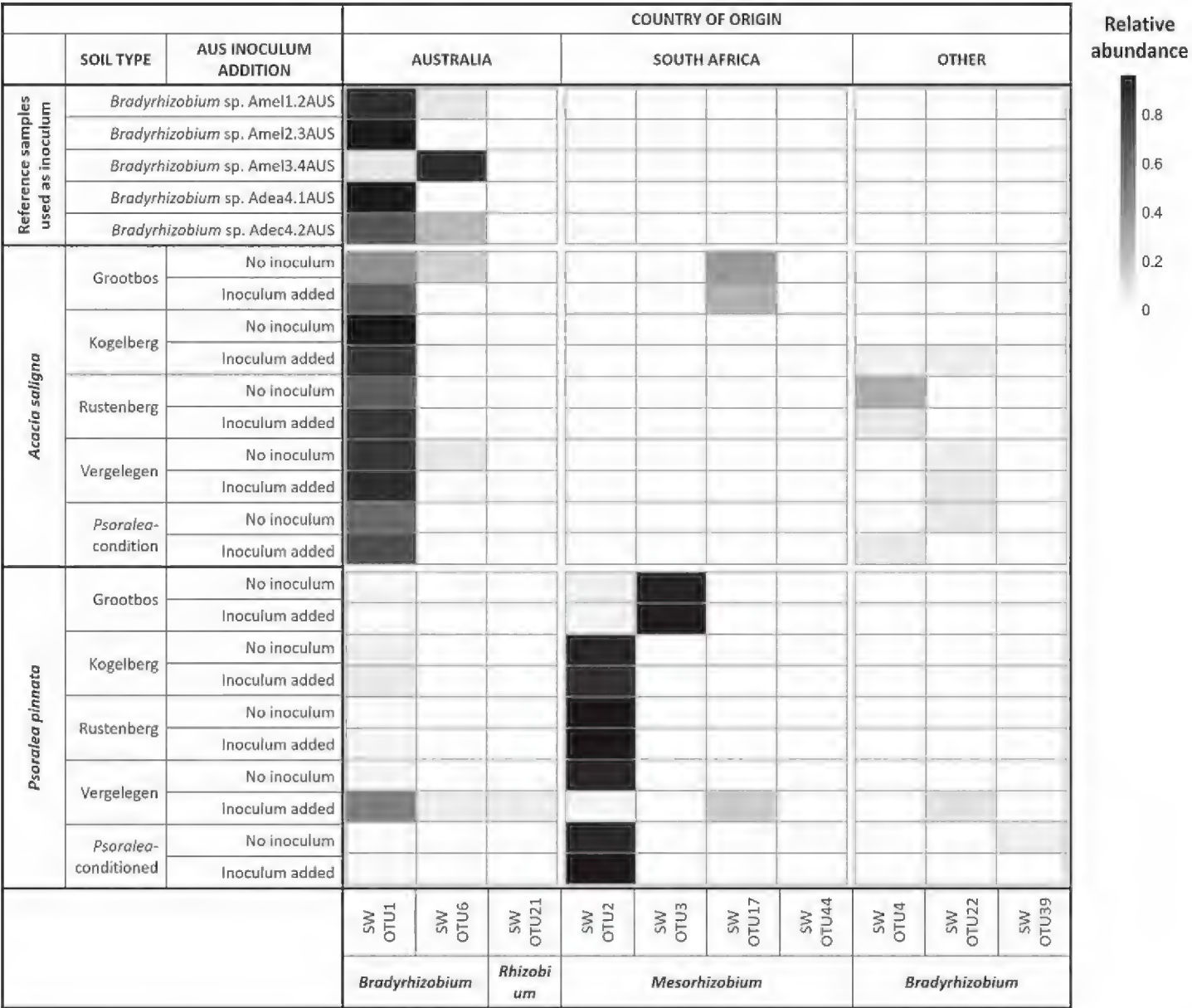
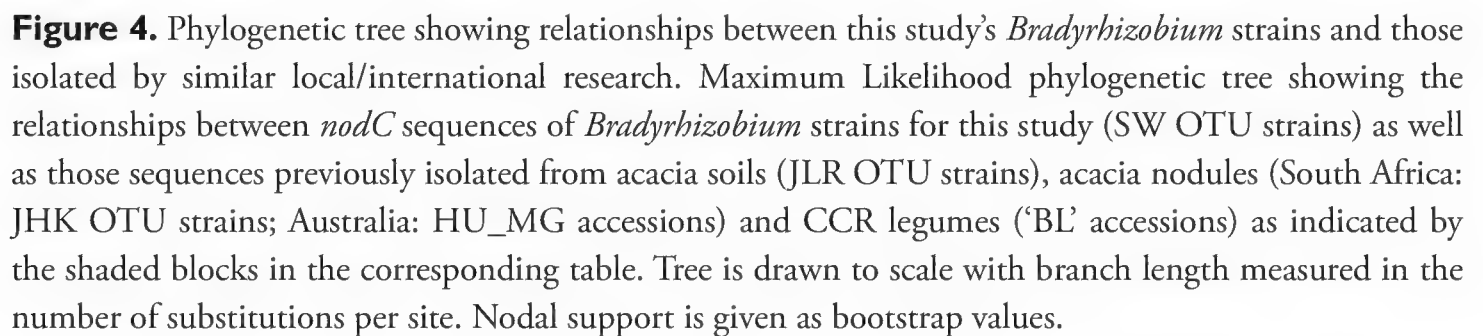


Figure 3. Relative abundances of the dominant OTUs associating with *Acacia saligna* and *Psoralea pinnata* seedlings. Heatmap based on the relative abundances of the ten rhizobial OTUs identified in this paper. Darker shades represent higher relative abundances. OTUs are arranged according to country of origin (top x-axis) based on blast results. Y-axis labels show the reference samples used as inoculum as well as the 20 species × soil × inoculum addition treatment combinations. OTU labels and genus identity based on blast results are given on the bottom x-axis.

Root nodule rhizobial composition comparisons

The relative abundances of the two dominant OTUs, SW OTU1 (259,830 sequence reads) and SW OTU6 (10,540 sequence reads), found in the reference samples, did not differ in *A. saligna* root nodules between the two inoculum treatments (SW OTU1: Paired t-test, $t_{(5)} = 1.034$, $p = \text{ns}$; SW OTU6: Wilcoxon signed-rank test, $W = 11$; $p = \text{ns}$). PERMANOVA indicated that Australian inoculum addition did not significantly change the relative composition of nodule OTU communities ($F_{(1,16)} = 0.405$; $p = \text{ns}$). However, the composition of nodule OTU communities differed significantly between host plant species ($F_{(1,16)} = 21.485$, $p < 0.001$) (Suppl. material 1: Table S8). Post-hoc analysis using the *simper* function showed that this significant host species effect was largely driven by SW OTU1 and SW OTU2 which accounted for 35.35%



and 34.01%, respectively, of the total compositional dissimilarity in nodule rhizobial communities between the two legume species (Suppl. material 1: Table S9). Specifically, *A. saligna* associated predominantly with *Bradyrhizobium* SW OTU1, while *P. pinnata* predominantly associated with *Mesorhizobium* SW OTU2. The remaining OTUs each accounted for less than 10% of the dissimilarity of root nodule communities between the two legume species (Fig. 3; Suppl. material 1: Table S9).

Discussion

Australian acacias have been co-introduced with their *Bradyrhizobium* strains into several regions across the globe (Rodríguez-Echeverría 2010; Crisóstomo et al. 2013; Warrington et al. 2019), and these exotic bradyrhizobia have the potential to negatively impact native legumes by outcompeting native rhizobia or by forming ineffective novel associations with them (Rodríguez-Echeverría et al. 2012; Le Roux et al. 2017a). In this study, however, we reject our hypothesis that the presence of exotic Australian bradyrhizobia negatively impacts the native CCR legume, *Psoralea pinnata*, as this species is successful at sanctioning these bacteria in favour of its familiar native *Mesorhizobium* strains. Whether the opposite is true for invasive Australian *Acacia saligna* (i.e., increased performance due to familiar associations) cannot be wholly resolved here as it formed familiar associations with Australian bradyrhizobia in all soils, with only one instance of a novel association. This suggests that the limited effects of inoculum addition on performance is likely due to the known widespread presence of exotic Australian bradyrhizobia in acacia-invaded CCR soils (Ndlovu et al. 2013; Keet et al. 2017; Le Roux et al. 2018; Warrington et al. 2019). Our study suggests that these exotic bradyrhizobia are also present in pristine, uninvaded, CCR soils.

Recent evidence suggests that nodule communities are largely made up of so-called core microbiomes, consisting of the most compatible and effective symbionts of the host (Shade and Handelsman 2012; Rodríguez-Valdecantos et al. 2017), likely as a result of host selection coupled with neutral processes such as ecological drift (Ramoneda et al. 2020). Although these mechanisms were not explicitly tested here, our NGS results and OTU comparisons suggest that *Bradyrhizobium* and *Mesorhizobium* are the core symbionts (through host selection) of *A. saligna* and *P. pinnata*, respectively (for *A. saligna* also see: Marsudi et al. 1999; Lafay and Burdon 2001; Keet et al. 2017; and for *P. pinnata* also see: Kanu and Dakora 2012; Lemaire et al. 2015). It is likely that the predominant association of *P. pinnata* with *Mesorhizobium*, and its ability to successfully sanction exotic *Bradyrhizobium* associations, resulted in the negligible effect of Australian bradyrhizobia on its performance. Although the exact mechanisms are not known in this case, legumes are known to minimise the impact of ineffective rhizobial associations through partner selection (Heath and Tiffin 2009; Sachs et al. 2010) and sanctioning (Kiers et al. 2003; Sachs and Simms 2006). Additionally, our results show that CCR *Mesorhizobium* strains can co-exist with Australian bradyrhizobia. Both

Mesorhizobium and *Bradyrhizobium* have adaptations to seasonally dry, acidic soils, likely resulting in overlapping distributions (Rodríguez-Echeverría et al. 2003; Dlodlu et al. 2018a). In contrast, other native CCR legumes have been found to be unable to limit associations with novel, and potentially less effective, Australian *Bradyrhizobium* strains in invaded sites (Le Roux et al. 2016). *Psoralea pinnata* is often one of the few native CCR legumes to regenerate through passive restoration in sites where Australian acacias have been cleared (Reinecke et al. 2008), highlighting its ability to survive in *Bradyrhizobium*-enriched soils. Therefore, the impact of co-invading acacias and bradyrhizobia is likely to be negligible on this native legume, at least from a nitrogen-fixing symbiosis perspective, making it a good candidate for active restoration.

Aside from the prevalence of familiar rhizobial associations, both *A. saligna* and *P. pinnata* formed a single novel association within Grootbos and Vergelegen soils, respectively. When grown in Grootbos soils, *A. saligna* plants had nodules containing high relative abundances of *Mesorhizobium* SW OTU17 regardless of inoculum treatment (Fig. 4; Suppl. material 1: Table S10), one of only two non-*Bradyrhizobium* associations identified for this species in this study. In Vergelegen soils, when Australian inoculum was not added, *P. pinnata* associated with the dominant *Mesorhizobium* SW OTU2. However, associations predominantly involved the *Bradyrhizobium* SW OTU1 when Australian inoculum was added. Nonetheless, both legume species had similarly poor growth performances under both Grootbos and Vergelegen soils, even when forming familiar associations. Therefore, while it is intuitive to attribute the poor growth performances to these novel rhizobial associations, which appear to be potentially bordering on parasitism (Denison and Kiers 2004; Rodríguez-Echeverría et al. 2012), the similarly poor seedling performances in these soils, even when forming familiar associations, suggests that site-specific edaphic conditions may have played a greater role.

Overall, differences in soils, rather than inoculum addition, largely explained differences in the growth performance and BNF proxies (i.e., $\delta^{15}\text{N}$ values and nodule numbers) of both legume species (Fig. 1). Both species generally performed poorly in Grootbos, Kogelberg and Vergelegen soils and performed best in Rustenberg and *Psoralea*-conditioned soils, regardless of inoculum addition or associations with preferred rhizobia (Fig. 1, 3). Our rhizobial efficacy results (Fig. 2) showed that $\delta^{15}\text{N}$ values of both species decreased with increased nodulation, suggesting BNF is occurring, which is also suggested by near zero $\delta^{15}\text{N}$ values at some sites (Fig. 1). However, importantly, neither the nodule number– $\delta^{15}\text{N}$ relationship, nor $\delta^{15}\text{N}$ values themselves, were significantly influenced by Australian inoculum addition, but $\delta^{15}\text{N}$ values did differ across sites (Fig. 1). Therefore, these differences in seedling performance we observed are likely due to differences in soil-specific abiotic properties, such as differences in nutrient levels or water-holding capacity and/or differences in biotic conditions such as pathogen loads (Thrall et al. 2007). While we did not test for differences in these soil characteristics between our study sites, previous research illustrates that these are likely to be present in our soils. Keet et al. (2021) sampled soils throughout the CCR and

found pH, bioavailable inorganic phosphorus, total carbon, nitrate-nitrogen (NO_3^-), ammonium-nitrogen (NH_4^+) and total available nitrogen to differ significantly among all sampled sites. Importantly, Keet et al.'s (2021) study was conducted at similar spatial scales to ours and at sites near ours. Such strong spatial turnover in soil abiotic conditions is characteristic of CCR habitats (Linder 2003, 2005) and likely impacts both soil microbial (e.g., Keet et al. 2019) and plant (see Ellis et al. 2014) communities. The overall effects of soil type on plant performance that we observed here, therefore, likely reflect major differences in soil abiotic conditions and their knock-on effects on soil microbial communities, between sites. Also, while we did not quantify differences in nitrogen isotopic fractionation between soils, such differences may explain the differences in $\delta^{15}\text{N}$ values we observed for both legume species between sites.

While we cannot completely exclude the possibility that cross-contamination explains the dominance of the same *Bradyrhizobium* strains in *A. saligna* root nodules of seedlings grown in inoculated and uninoculated soils, several considerations suggest that this is an unlikely explanation. Firstly, there was a significant overall inoculation effect for *A. saligna* seedlings for many performance measurements. This was never the case for *P. pinnata* seedlings. Secondly, stringent protocols to minimise cross-contamination were put in place during soil collection and processing, inoculation applications and the glasshouse experiment (watering, etc.). Furthermore, Keet et al. (2017) sequenced root nodule communities from 19 different Australian *Acacia* species (including *A. saligna*) sampled across a wide geographic range (up to 900 km apart) in South Africa. They used the same DNA barcode as we did and found that all acacias shared a few, but highly abundant, *Bradyrhizobium* OTUs. The most dominant OTU identified by them, JHK OTU1, comprising 49% of their 98,000 sequence reads, is also the most dominant *Bradyrhizobium* strain in this study, SW OTU1 (Fig. 4). More recently, Le Roux et al. (2018) characterised rhizobial communities in acacia-invaded (by six different species, including *A. saligna*) and uninvaded CCR soils. They found that dense acacia stands homogenised rhizobial community structure and enriched soils for *Bradyrhizobium* strains. The most dominant *Bradyrhizobium* OTU identified by Le Roux et al. (2018) from acacia-invaded soils was JLR OTU1, comprising almost 10% of the 99,600 sequence reads they obtained, which corresponds to strains previously isolated from acacias in Australia (UH_MG accessions; Urbina and Klock, unpublished) and Keet et al.'s (2017) JHK OTU1, and thus our SW OTU1 (Fig. 4). Additionally, both our Rustenberg Winery and Vergelegen Wine Farm sites were also previously sampled by Le Roux et al. (2018). These authors found relative abundances of *Bradyrhizobium* strains isolated from paired invaded and uninvaded areas at these two sites to be similar (Le Roux et al. 2018). Together with the known history of co-introduction of Australian *Bradyrhizobium* and acacias to South Africa (Ndlovu et al. 2013; Warrington et al. 2019), these findings strongly suggest that the most parsimonious explanation for the dominance of the same Australian *Bradyrhizobium* in *A. saligna* nodules between our treatments is that they are, in fact, already established and widespread in CCR soils.

Conclusion

This study adds to a growing body of evidence suggesting that rhizobial mutualist availability is no longer a major limiting factor for acacia invasion (see Wandrag et al. 2020 and references therein) as exotic Australian *Bradyrhizobium* strains appear to be established within pristine CCR soils (Ndlovu et al. 2013; Warrington et al. 2019). Additionally, the one novel *Mesorhizobium* association for *A. saligna* in this study did not hamper seedling performance, and similar associations with *Mesorhizobium* strains have been previously documented (Amrani et al. 2010; Boukhatem et al. 2012; Crisóstomo et al. 2013). Australian acacias have the ability to utilise a few (often co-introduced) *Bradyrhizobium* strains interchangeably (Wandrag et al. 2013; Keet et al. 2017; Warrington et al. 2019). Therefore, co-introduction of effective rhizobial partners and mutualist promiscuity are not mutually exclusive but may act synergistically to enhance acacia colonisation success and invasiveness. Consequently, the presence of Australian bradyrhizobia in pristine CCR soils suggests that this region is highly susceptible to further acacia invasion with potentially detrimental above- and belowground biodiversity impacts. The CCR is home to a high diversity of rhizobia (Le Maitre et al. 2011; Sprent et al. 2017; Dlodlu et al. 2018a) which is tightly linked to legume community composition (Slabbert et al. 2010; Lemaire et al. 2015; Le Roux et al. 2016). While there were no notable negative impacts of exotic bradyrhizobia on the *Mesorhizobium* strains identified in this study, the same may not be true for other rhizobial genera or their legume hosts (Le Roux et al. 2016). Our results suggest that mutualisms with belowground microbiota can contribute more towards habitat invasibility than previously thought.

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Supplementary material I

Supplementary materials

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Data type: docx

Explanation note: Electronic Supplementary Materials (ESM1, ESM2). Tables S1–S9. Figures S1–S4.

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